Properties of myofibril-bound calpain activity in longissimus muscle of callipyge and normal sheep[1]

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ABSTRACT: Properties of the calpain bound to myofibrils in longissimus muscle from callipyge or noncallipyge sheep were examined after 0, 1, 3, and 10 d of postmortem storage at 4°C. Western analysis has shown that most of this calpain is μ-calpain, although the sensitivity of the antibodies used in the earlier studies could not eliminate the possibility that up to 10% of the calpain was m-calpain. The calpain is bound tightly, and very little is removed by washing with the detergent Triton X-100; hence, it is not bound to phospholipids in the myofibril. Over 25% of total μ-calpain was bound to myofibrils from at-death muscle, and this increased to ~40% after 1 d postmortem. The amount of myofibril-bound μ-calpain increased only slightly between 1 and 10 d of postmortem storage. The percentage of autolyzed μ-calpain increases with time postmortem until after 10 d postmortem, when all myofibril-bound μ-calpain is autolyzed. The specific activity of the myofibril-bound calpain is very low and is only 6 to 13% as high as the specific activity of extractable μ-calpain from the same muscle. It is unclear whether this low specific activity is the result of unavailability of the active site of the myofibril-bound calpain to exogenous substrate. The myofibril-bound calpain degrades desmin, nebulin, titin, and troponin T in the myofibrils, and also releases undegraded α-actinin and undergoes additional autolysis when incubated with Ca^{2+}; all these activities occurred slowly considering the amount of myofibril-bound calpain. Activity of the myofibril-bound calpain was partly (58 to 67%) inhibited by the calpain inhibitors, E-64 and iodoacetate; was more effectively inhibited by a broader-based protease inhibitor, leupeptin (84 to 89%); and was poorly inhibited (43 to 45%) by calpastatin. Release of undegraded α-actinin and autolysis are properties specific to the calpains, and it is unclear whether some of the myofibril-bound proteolytic activity originates from proteases other than the calpains or whether the active site of myofibril-bound calpain is shielded from the inhibitors. Activities and properties of the myofibril-bound calpain were identical in longissimus muscle from callipyge and normal sheep, although previous studies had indicated that the "normal" longissimus was much more tender than the callipyge longissimus. Hence, it seems unlikely that the myofibril-bound calpain has a significant role in postmortem tenderization of ovine longissimus.

Key Words: Calpains, Myofibrils, Sheep Breeds

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Introduction

Several studies have indicated that, in some instances, proteolysis of some of the proteolytically most sensitive cytoskeletal/myofibrillar proteins, such as nebulin, desmin, and troponin T, is not detected in bovine skeletal muscle until 3 d of postmortem storage at 2°C, even when using sensitive Western analysis (Ho et al., 1996, 1997; Huff-Lonergan, 1996). After 24 h of postmortem storage at 2 to 4°C, muscle pH has fallen to 5.8 or less and muscle temperature to less than 6°C

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(Koohmaraie et al., 1987). The optimum pH for activity of either μ- or m-calpain or their autolyzed products (Edmunds et al., 1991) is 7.4 to 7.6, and the rate of hydrolysis of a casein substrate is threefold faster at 25°C than at 0% C (Dayton et al., 1976). Proteolytic activity of μ-calpain as assayed in vitro decreases rapidly during postmortem storage to 18 to 48% of its initial activity within 2 d postmortem (Vidalenc et al., 1983; Koohmaraie et al., 1987; Boehm et al., 1998). Consequently, μ-calpain activity in muscle at 2 d postmortem is ~1 to 4% of its at-death activity. Furthermore, although the intracellular free Ca^{2+} is likely to be 100 μ*M* or higher at 2 d postmortem (Parrish et al., 1981; Jeacocke, 1993), sufficient for maximal activity of μ-calpain in in vitro assays, 2 d postmortem muscle still has active calpastatin (Vidalenc et al., 1983; Koohmaraie et al., 1987; Boehm et al., 1998) that could inhibit any residual μ-calpain activity.

A significant amount of total muscle μ-calpain becomes associated with the myofibrillar fraction in bovine muscle during postmortem storage (Geesink and Goll, 1995). Membrane- and myofibril-bound μ-calpain is not inhibited by calpastatin (Hatanaka et al., 1984; Boehm et al., 1998). We investigated myofibrils from callipyge and normal sheep to learn whether μ-calpain is also bound to myofibrils in postmortem sheep muscle and whether there are any differences in myofibril-bound calpain in callipyge and normal sheep.

Materials and Methods

Animals. The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of the animals in this study. Ten Dorset sheep, averaging 3 yr of age, all ewes, and five of them carriers and five of them noncarriers of the callipyge gene as assessed by leg score, were slaughtered in pairs (one callipyge and one noncallipyge). Leg score was used because at the time when this study was done, visual assessment was the most accurate method for segregating sheep into normal and callipyge groups. Average carcass and muscle weights of these animals have been reported (Delgado et al., 2001).

Muscle Sampling. The longissimus muscle was removed from the left side of the carcass within 30 min after exsanguination for the at-death samples; longissimus muscle was taken from the right side of the carcass after 24 h at 4°C and placed at 4°C until sampled. Ten- to 15-g samples were removed from the at-death muscle and from the muscle after 1, 3, and 10 d of storage at 4°C, and the samples were minced and then were frozen at –70°C for 8 to 10 mo before 2-g aliquots were removed for preparation of myofibrils. Myofibrils were made by homogenizing the minced muscle in 3 vol (vol/wt) of 100 m*M* Tris, 10 m*M* EDTA, 10 m*M* β-mercaptoethanol (**MCE**), adjusted to pH 8.3 with HCl, and the cocktail of protease inhibitors described previously (Edmunds et al., 1991). The homogenate was centrifuged at 37,500 × g_{max}, and the pellet was suspended in 10 vol (vol/wt, based on original weight of the aliquot) of a standard salt solution (100 m*M* KCl, 20 m*M* potassium phosphate, 2 m*M* $MgCl_2$, 2 m*M* EGTA, 1 m*M* NaN_3, pH 5.8) by using a 10-s burst with a Polytron homogenizer (setting of 6; medium speed). The homogenate was centrifuged at 1,000 × g_{max} for 10 min, and the pellet resuspended by using a Polytron as before. The washing step was repeated twice, protein concentration of the final suspension of thrice-washed myofibrils was determined by using the biuret procedure (Gornall et al., 1949), and the myofibrils were stored in standard salt solution at 4 to 8°C for 24 h or less.

Assays of Myofibril-Bound Calpain Activity. Myofibril-bound calpain had very low activity, so it was necessary to use ^{14}C-labeled casein as a substrate to enable detection of the calpain activity. Labeling was done by using reductive alkylation with ^{14}C-formaldehyde and sodium cyanoborohydride (Dottavio-Martin and Raven, 1978). Hammersten-casein was dissolved in 50 m*M* Tris-HCl, pH 7.0, to a final concentration of 10 mg in 20 mL. Two hundred fifty microliters of ^{14}C formaldehyde (1 mCi/mL) was added to 3 mL of casein followed by addition of 1 mL of 150 mg/mL sodium cyanoborohydride that had been freshly dissolved in 50 m*M* Tris-HCl, pH 7.0. The mixture was incubated for 6 h at 25°C with shaking in a water bath to allow labeling. The labeled casein was dialyzed against a 100-fold (vol/vol) excess of 50 m*M* Tris-HCl, pH 7.5, 1 m*M* NaN_3 for 2 d with two changes per day, or until the dialysate had less than 140 counts·min^{-1}·mL^{-1}. After dialysis, 10-μL aliquots were added to 5 mL of scintillation fluid (ScintiVerse, Fisher Scientific) to determine the degree of labeling, and the protein content of the labeled casein was determined by using the BCA procedure (Smith et al., 1985). The labeled casein (1.64 mg casein/mL) had 2,068 cpm/μg casein, and was stored in frozen 1-mL aliquots at –30°C until used.

Several experiments were done to calibrate the ^{14}C-casein assay. Purified μ-calpain from bovine lung was diluted sequentially in dilution buffer, and activity of the diluted samples was measured in the standard calpain assay (Delgado et al., 2001), except counting ^{14}C-labeled peptides soluble in 2.5% TCA instead of using absorbance at 280 nm. The ^{14}C-casein assay could detect 100 ng of calpain and gave a linear response to 600 ng of μ-calpain when assayed in a 60-min assay (Figure 1). The activity of identical samples of purified μ-calpain was determined by using either the standard assay used in the laboratory (Delgado et al., 2001) or the ^{14}C casein assay. The average of three such experiments showed that 1 unit of calpain activity in the standard assay was equivalent to 2.7×10^6 cpm in the ^{14}C-casein assay (standard error was 0.13×10^6 cpm).

Determination of myofibril-bound calpain activity was done in microcentrifuge tubes. The final conditions of the assay were as follows: 370 μg of myofibrillar protein in 92.5 μL of standard salt solution (100 m*M* KCl, 20 m*M* potassium phosphate, pH 7.2, 2 m*M* $MgCl_2$, 2 m*M* EGTA, 1 m*M* NaN_3), 28.7 μL of standard salt

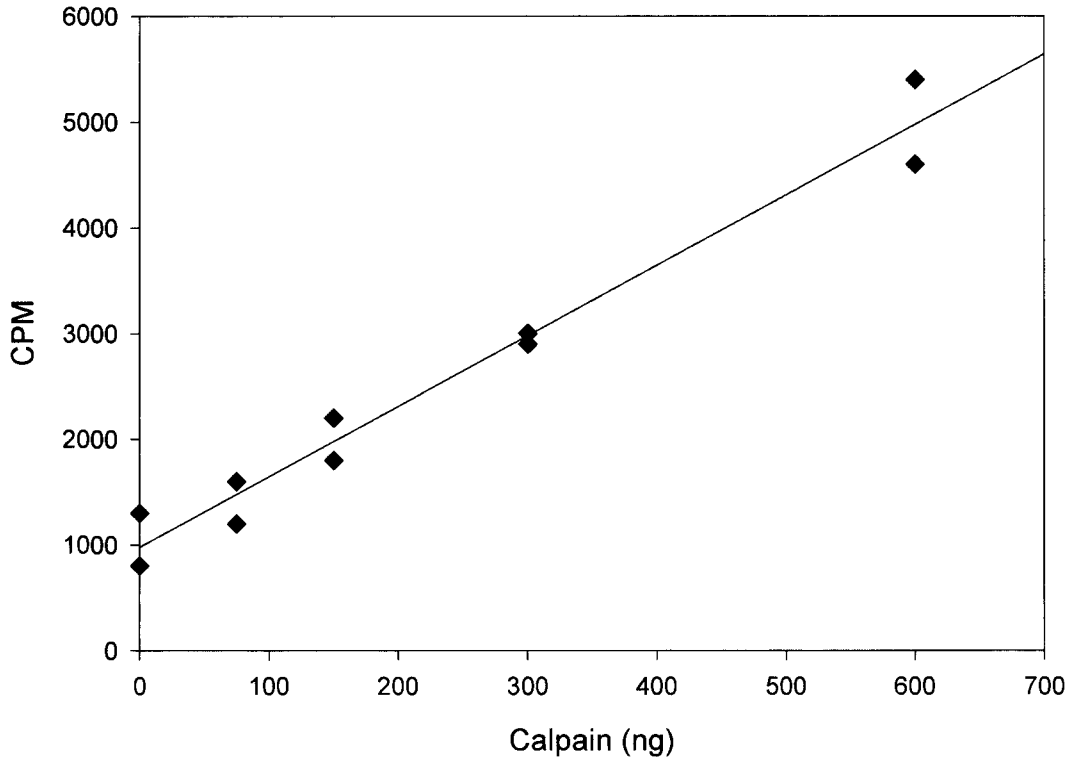

Figure 1. Relationship between concentration of purified μ-calpain and response of the ^{14}C-casein assay. Conditions: different amounts of purified μ-calpain from bovine lung were incubated in 75 m*M* Tris-HCl, pH 7.5, 0.25 m*M* EDTA, 10 m*M* MCE, and 3.5 mg ^{14}C-casein for 60 min at 25°C. Activity was determined by measuring the release of trichloroacetic acid-soluble peptides containing ^{14}C as described in Materials and Methods. The results of two separate experiments are shown; a least squares regression analysis was used to produce the plot.

solution, 5 μL of MCE added just before the assay, 25 μL of ^{14}C-labeled casein (1.64 mg protein/mL), and 16.8 μL of 100 m*M* $CaCl_2$ to make a final volume of 168 μL. Incubation was done with shaking for 48 h at 15°C to detect the small amount of myofibril-bound calpain activity present. The 15°C incubation was used to retard microbial growth during the long incubation time used. Because of the long assay times, all experiments included four control assays: 1) a tube incubated for 0 min with standard salt solution in place of the Ca^{2+}; 2) a tube incubated for 0 min with 10 m*M* Ca^{2+} present (these two controls were used to determine the basal, 0-time blanks; the two measurements were always the same or very similar and were in the range of 800 to 1000 cpm); 3) a tube incubated for 48 h containing all the ingredients that the sample tubes had but with 16.8 μL of 2 m*M* EGTA (final concentration = 0.2 m*M*) in place of the Ca^{2+} (a Ca^{2+}-free blank); and 4) a positive control containing 150 ng of purified μ-calpain added in 5 μL and containing 23.7 μL instead of 28.7 μL of standard salt solution. After 48 h at 15°C, the myofibril suspensions were sedimented at $16{,}000 \times g_{max}$ for 5 min, 155 μL of the supernate was removed and added to 50 μL of bovine serum albumin (10 mg of protein/mL), and 205 μL of 10% cold trichloroacetic acid (**TCA**). The undigested casein was removed by centrifugation at $16{,}000 \times g_{max}$ for 10 min, the supernate was transferred quantitatively to a scintillation vial, and 5 mL of scintillation fluid (ScintiVerse) was added. Counting was done for 5 min.

Preliminary experiments indicated that under the conditions of our assay (4 mg of myofibrillar protein per milliliter and 15°C), release of ^{14}C-labeled peptides increased nearly linearly with up to 48 h of incubation (Table 1), so we chose 48 h instead of 24 h of incubation. We did not attempt to extend the incubations to yet

Table 1. Effect of incubation time on the release of trichloroacetic acid-soluble peptides from casein by myofibril-bound protease[a]

Source of myofibrils (d postmortem)	Time of incubation, h	
	24	48
0	2,034[b]	4,954
1	3,720	6,896
3	4,546	9,395
10	4,131	8,547

[a]Myofibrils were prepared from longissimus muscle and were washed three times as described in the Materials and Methods. Assays were done as described in the Materials and Methods; numbers are total counts per minute obtained after 5 min of counting and subtraction of the Ca^{2+}-free blanks.

[b]Numbers are means of two determinations done on each of six animals (12 total assays per mean).

longer times because of the problems inherent with very long incubation times when measuring enzyme activity (bacterial action, degradation of the myofibril-bound calpain, etc.). Other preliminary experiments showed that release of ^{14}C-labeled peptides from casein by myofibril-bound calpain increased linearly under the conditions used in this study up to 4 mg of myofibrillar protein per milliliter and then reached a plateau at higher myofibril concentrations. Therefore, subsequent studies used 4 mg of myofibrillar protein per milliliter in the assays.

Effect of Inhibitors on Myofibril-bound Calpain Activity. To learn more about the nature of the proteolytic activity associated with myofibrils, myofibril-bound proteolytic activity was determined in the presence of four inhibitors: 1) E-64 [epoxysuccinyl-L-,leucylamido-(4-guanidino)butane], an irreversible inhibitor of cysteine proteases, and an effective inhibitor of the calpains; 2) leupeptin (N-acetyl-L-Leu-L-Leu-L-Argininal), a transition state analog that inhibits a number of cysteine and serine proteases, including the calpains; 3) iodoacetate, an irreversible inhibitor of cysteine proteases, including the calpains; and 4) calpastatin, the intracellular protein that is specific for inhibition of the calpains. Conditions of the assays were the same as those described in the preceding paragraph except the 28.7 μL of standard salt solution contained a specified inhibitor at a given concentration. The final concentration of each inhibitor in the assay was 1) 0.33 m*M* E-64, 2) 1.50 m*M* leupeptin, 3) 0.41 m*M* iodoacetate, and 4) 0.4 unit calpastatin. One calpastatin unit is defined as the amount of calpastatin required to inhibit one unit of calpain assay in a standard casein assay (Koohmaraie, 1990). The calpastatin used in these studies was purified from bovine skeletal muscle and migrated with a relative molecular weight of 125,000 g/mol in SDS-PAGE. The inhibitor experiments used only myofibrils that had been prepared from 1-d postmortem longissimus muscle because Western analysis showed no significant increase in the amount of myofibril-bound calpain between 1 d and 10 d of postmortem storage (see figure 4, Delgado et al., 2001). Although myofibril-bound calpain *activities* are higher at 3 d than at 1 d in Table 1, this difference is small, and the 1-d time was chosen to minimize possible complications, such as the bacterial contamination that might occur with longer times of postmortem storage. The inhibitor experiments also used samples from three callipyge and three normal animals instead of five animals of each phenotype.

Earlier studies had indicated that most of the calpain associated with myofibrils in postmortem muscle was μ-calpain (Geesink and Goll, 1995; Boehm et al., 1998; Delgado et al., 2001). Neither m-calpain nor calpastatin could be detected in Western analyses of postmortem myofibrils, although the sensitivity of the antibodies used did not eliminate the possibility that both m-calpain and calpastatin could be present in postmortem myofibrils at levels less than 10% of the μ-calpain present (Boehm et al., 1998). Therefore, dot-blot analysis was used in an attempt to estimate the percentage of total muscle μ-calpain bound to the myofibrillar fraction in postmortem ovine muscle. The analyses used the supernate and pellet fractions obtained by fractionation of longissimus from callipyge and normal phenotypes (see figure 2 in Delgado et al., 2001) and monoclonal antibodies specific for μ-calpain. Replicate analyses of the same samples were variable; 28 to 44% of total μ-calpain was bound to the myofibril at d 0; 36 to 44% of total μ-calpain was bound to the myofibril at d 1; 43 to 60% of total μ-calpain was bound to the myofibril at d 3; and 47 to 69% of total μ-calpain was bound to the myofibril after 10 d of postmortem storage. Assuming that 40% of total muscle μ-calpain is bound to the myofibril after 1 d of postmortem storage, and that the dot-blot assays provided valid estimates of the approximate amount of μ-calpain bound to the myofibrils, the 1-d myofibrils contained approximately 0.13 μg of μ-calpain per milligram of myofibrillar protein. Thus, the 370 μg of myofibrillar protein in the assay tube contained ~48.1 ng, or ~0.437 pmol (2.6 n*M*), of μ-calpain whose activity could be detected only by long incubation times.

The assay mixtures containing the inhibitors but no Ca^{2+} were preincubated in standard salt solution for 42 h at 5°C on a rocking platform (Red Rocker, Hoefer Scientific Instruments, San Francisco, CA) to allow complete penetration of the inhibitors. Calcium was then added to a final concentration of 10 m*M*, and the tubes where incubated for an additional 48 h at 15°C. The same four control tubes described in the preceding paragraph were included with each assay, except that the two 0-time blanks were obtained by 1) sedimenting the myofibrils before the 42-h preincubation and 2) incubating the myofibrils plus inhibitor for 42 h and then sedimenting the myofibrils. The Ca^{2+} blank was preincubated for 42 h with the inhibitors and then 16.8 μL of 2 m*M* EGTA, added before incubating for another 48 h at 15°C. Also, for the positive control, the μ-calpain (5 μL of 30 ng protein/μL) was preincubated for 42 h before addition of 16.8 μL of 100 m*M* Ca^{2+}.

SDS-PAGE and Western Blotting. Samples for SDS-PAGE and Western analysis used separate microcentrifuge tubes treated identically to those used for assays of proteolytic activity. This was done to remove the potentially confounding effects that the presence of casein in the assays might have on endogenous degradation of the myofibrillar proteins. Final conditions for the tubes for SDS-PAGE were as follows: 3.76 mg of myofibrillar protein in 470 μL (8 mg myofibrillar protein/mL) of standard salt solution; 129.8 μL of standard salt solution; 5 μL of MCE, added just before use; and 67.2 μL of 100 m*M* $CaCl_2$ to make a final volume of 672 μL. The same four controls used in the activity assays were also included for the SDS-PAGE assays. After incubation at 15°C for 48 h, the samples were centrifuged at $16{,}000 \times g_{max}$ for 5 min. The pellet (myofibrils) was suspended in standard salt solution by using a

glass rod. Two volumes each of the supernate and of the suspended pellet were mixed with 1 vol of SDS-sample buffer (**SDS-SB**) containing 62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 0.5% MCE. After dissolution in SDS-SB, an aliquot of each sample was precipitated in 60% TCA, and protein concentration in the samples was determined as described by Karlsson et al. (1994).

Samples containing the protease inhibitors were prepared for SDS-PAGE in the same manner as described in the preceding paragraph, except that the 672-μL samples containing the protease inhibitors were preincubated for 42 h at 5°C before being incubated at 15°C for 48 h.

The SDS-PAGE was done according to the method of Laemmli (1970) using either 8- × 10-cm minigels or 14- × 17.7-cm gels. For the μ-calpain samples, the gels were 8% acrylamide; for α-actinin, desmin, and troponin T, the minigels were 10% acrylamide; the minigels for nebulin were 6% acrylamide; and the minigels for titin were 12.5% acrylamide. All gels contained 0.0267% bis-acrylamide as a percentage of the acrylamide present. The higher percentage acrylamide was used for the titin gels in an effort to detect the titin fragments at the expense of being able to monitor degradation of the intact titin polypeptide.

The separated polypeptides were transferred in 25 m*M* Tris-HCl (pH 8.2), 193 m*M* glycine and 15% methanol to either Hybond-C nitrocellulose or Hybond-P polyvinylidine fluoride (**PVDF**) membranes for 2.5 h at 2 to 4°C and 200 to 300 mA by using a wet-transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Blocking and staining with antibodies was done as described previously (Delgado et al., 2001). The primary antibodies used were as follows: anti-α-actinin, monoclonal clone EA-53 (Sigma Chem. Co., St. Louis, MO), 1:1000 dilution; anti-calpastatin, polyclonal, MARC-USDA (Doumit and Koohmaraie, 1999), 1:500 dilution; anti-μ-calpain, monoclonal, clone B2F9, MARC-USDA (Geesink and Koohmaraie, 1999b) 1:5 dilution; anti-desmin, monoclonal, cloneD3, Hybridoma Bank, 1:2 dilution; anti-nebulin, monoclonal, clone NB2, Sigma, 1:250 dilution; anti-titin, monoclonal, clone 9D10, Hybridoma Bank, 1:100 dilution; and anti-troponin T, monoclonal, clone CT3, Hybridoma Bank, 1:1,000 dilution. Blots were incubated with the primary antibodies for 60 min at room temperature (Delgado et al., 2001). The secondary antibody used was alkaline phosphatase conjugated to anti-mouse IgG (A-5153, Sigma Chem. Co.) at a 1:1,000 dilution for all Western analyses except the anti-calpastatin, which used alkaline phosphatase conjugated to anti-mouse IgG (Fab specific/A-179, Sigma Chem. Co.) also at a 1:1,000 dilution. Lanes containing molecular weight markers were stained with amido black (Bio-Rad Laboratories, Hercules, CA).

Materials. Acrylamide, ammonium sulfate, N,N′-methylene-bis-acrylamide, N,N,N′N′-tetramethylethylethylene, and the BCIP/NBT substrate for alkaline phosphatase were purchased from Bio-Rad Laboratories, Hercules, CA; bovine serum albumin, EGTA, iodoacetic acid, sodium salt, MCE, sodium azide, SDS, the filtered sheep serum for membrane blocking, ^{14}C-formaldehyde, and Tris were all purchased from Sigma, St. Louis, MO; $CaCl_2$ was from J. T. Baker, Phillipsburg, NJ; Hammersten casein was from United States Biochemical Corporation, Cleveland, OH; E-64 and leupeptin were purchased from Peptides International, Louisville, KY; and the Hybond-C and Hybond-P membranes were from Amersham Pharmacia Biotech, Piscataway, NJ. All other chemicals were reagent grade or purer. Deionized water was used in all experiments.

Results

Myofibril-Bound Calpain Activity. Previous studies have shown that myofibrils contain significant amounts of μ-calpain (Geesink and Goll, 1995; Boehm et al., 1998; Delgado et al., 2001), and that at least some of this calpain is proteolytically active (Boehm et al., 1998). As indicated earlier, most and perhaps all the myofibril-bound calpain activity is from μ-calpain, although we cannot eliminate the possibility that up to 10% of the total activity originates from m-calpain. Therefore, the myofibrillar-bound proteolytic activity will be referred to as "calpain activity" in this article. Also, the assays were done on myofibrils that had been frozen at –70°C. Freezing of muscle tissue does not affect either calpain or calpastatin activity, and similar myofibril-bound calpain activities have been obtained using myofibrils from unfrozen muscle (Boehm et al., 1998). Hence, it is unlikely that freezing had any significant effect on our results. Myofibril-bound proteolytic activity in sheep longissimus muscle increased by 56 to 74% during the first day of postmortem storage and then remained nearly constant during the next 9 d postmortem (Table 2). These changes in myofibril-bound proteolytic activity parallel the changes observed in the dot-blot analyses, which indicated no significant increase in amount of μ-calpain bound to the myofibrillar fraction after 1 d postmortem. There was no difference between the callipyge and the normal sheep in the amount of myofibril-bound proteolytic activity, and, in general, no differences in myofibril-bound proteolytic activity were found between the callipyge and normal sheep in this study. Therefore, data from these two groups were combined in some experiments.

The myofibril-bound proteolytic activity is bound tightly to the myofibrils and is not removed by washing with 1% Triton X-100, followed by extensive washing to remove the Triton (Table 3). Boehm et al. (1998) also found that the proteolytic activity bound to bovine skeletal muscle myofibrils was not removed by Triton X-100. Although it has been widely suggested that the calpains bind to a phospholipid in cell membranes or organelles and are activated by such binding (Suzuki et al., 1990; Saido et al., 1992), failure to be extracted by Triton X-100 suggests that the calpain in myofibrils is not bound to a phospholipid.

Table 2. Changes in myofibril-bound proteolytic activity of callipyge and normal longissimus dorsi during postmortem storage[a]

	Time postmortem, d			
Phenotype	0	1	3	10
Callipyge	10.2[b]	17.9	16.5	17.2
Normal	11.8[b]	18.4	17.4	14.0

[a]Numbers are counts per minute of ^{14}C released from ^{14}C-labeled casein per microgram of myofibrillar protein after incubation with myofibrils for 48 h at 15°C (see Materials and Methods).
[b]Days postmortem within phenotype are significantly different ($P < 0.01$). Standard error was 1.2 counts.

Western analysis of myofibrils that had been incubated for 48 h at 15°C indicated that desmin, titin, and nebulin in the myofibrils were degraded after incubation for 48 h in the presence of Ca^{2+} (Figures 2 and 3). Nebulin was especially susceptible to degradation, with nebulin in the d-0, d-1, d-3, and d-10 myofibrils being nearly completely degraded after 48 h at 15°C to small polypeptides that were barely detectable. This degradation was Ca^{2+}-dependent. Titin also was completely degraded to peptides ranging approximately from 45 to 250 kDa after 48 h at 15°C (Figure 3). Degradation of desmin, on the other hand, was barely detectable after 48 h of incubation. It is unclear whether these differences are due to localization of the bound μ-calpain or to differences in susceptibility of nebulin, titin, and desmin to calpain degradation. Degradation of nebulin or titin would likely sever connections of the thin and thick filaments with the Z-disk and have significant effects of tenderness.

Effect of Inhibitors on Myofibril-Bound Proteolytic Activity. The different proteolytic inhibitors used in this study caused a 58 to 67% inhibition of the myofibril-bound proteolytic activity (Table 4). Assuming that the myofibrils in the assays in Table 4 had approximately 0.13 μg of μ-calpain bound per milligram of myofibrillar protein, the assays contained approximately 48.1 ng, or 2.6 n*M* μ-calpain. Consequently, the inhibitors were present in very large inhibitor/μ-calpain molar ratios:127,000:1 for E-64; 577,000:1 for leupeptin; and 158,000:1 for iodoacetate. It is surprising, therefore, that the myofibrillar-bound proteolytic activity was not completely inhibited. Concentration of purified μ-calpain in the positive control assays was 8.1 n*M*, and the large molar excess of inhibitors in the assays nearly completely inhibited the purified μ-calpain (Table 4), indicating that the inhibitors were active.

Table 3. Effect of washing with Triton X-100 on myofibril-bound proteolytic activity[a]

	Source of myofibrils, d postmortem	
Treatment[b]	0	10
No Triton wash	2,940	6,414
Triton X-100 wash	2,200	4,810

[a]Myofibrils were prepared from longissimus muscle. Assays were done as described in Materials and Methods; numbers are total counts per minute obtained after 5 min of counting and subtraction of the Ca^{2+}-free blank. Numbers are means of two determinations done on each of six animals (12 total assays per mean).
[b]Untreated myofibrils were prepared as described in Materials and Methods (three washes). The Triton X-100-washed myofibrils were washed twice in three volumes (vol/vol) of 1% Triton X-100, 100 m*M* KCl, 20 m*M* K-phosphate, pH 7.0, 2 m*M* $MgCl_2$; 2 m*M* EGTA, 1 m*M* NaN_3 followed by washing six times with 100 m*M* KCl to remove all detergent from the myofibrils.

Purified bovine skeletal muscle calpastatin in large excess also only partly inhibited the myofibril-bound proteolytic activity (Table 5). Assuming 2.6×10^6 cpm equals 1 unit of calpain activity, and using the average total cpm for an assay as 7531 cpm, it can be calculated that each tube contained approximately 2.79×10^{-3}, or 0.00279, units of calpain activity if all the myofibril-bound proteolytic activity originated from calpain. Hence, calpastatin was present in the assay at a ratio of 143 calpastatin:1 myofibril-bound calpain, based on units of activity (or at an even greater ratio if not all the myofibril-bound proteolytic activity originated from calpain). Again, it is surprising that the large excess of calpastatin did not completely inhibit the myofibrillar bound proteolytic activity, especially since it completely inhibited the purified μ-calpain, which was present in greater amounts than the myofibril-bound calpain (8.1 n*M* purified μ-calpain and approximately 2.6 n*M* myofibril-bound μ-calpain).

The SDS-PAGE of the myofibrils incubated for 48 h in the presence of inhibitors showed that the Ca^{2+}-induced degradation of desmin, titin, and troponin T was partly prevented by addition of the protease inhibitors (degradation of troponin T is shown in Figure 4). Moreover, autolysis of the myofibril-bound μ-calpain is enhanced by the addition of Ca^{2+} and is inhibited by the presence of E-64, leupeptin, and iodoacetate (Figure 5). The calpains are unique in that they release α-actinin from the Z-disks of myofibrils but do not degrade it (Goll et al., 1991). The SDS-PAGE of the supernate after sedimentation of the myofibrils showed that intact α-actinin was released from the myofibrils in the absence of E-64, leupeptin, and iodoacetate, and that this release was inhibited by the three inhibitors (Figure 6). Calpastatin, which is a specific inhibitor for the calpains and which was added in large excess in the assays, had less effect on total myofibril-bound proteolytic activity than the other three inhibitors (Table 5; 43 to 45% calpastatin inhibition compared with 58 to 67% inhibition by the other three inhibitors) and had very little

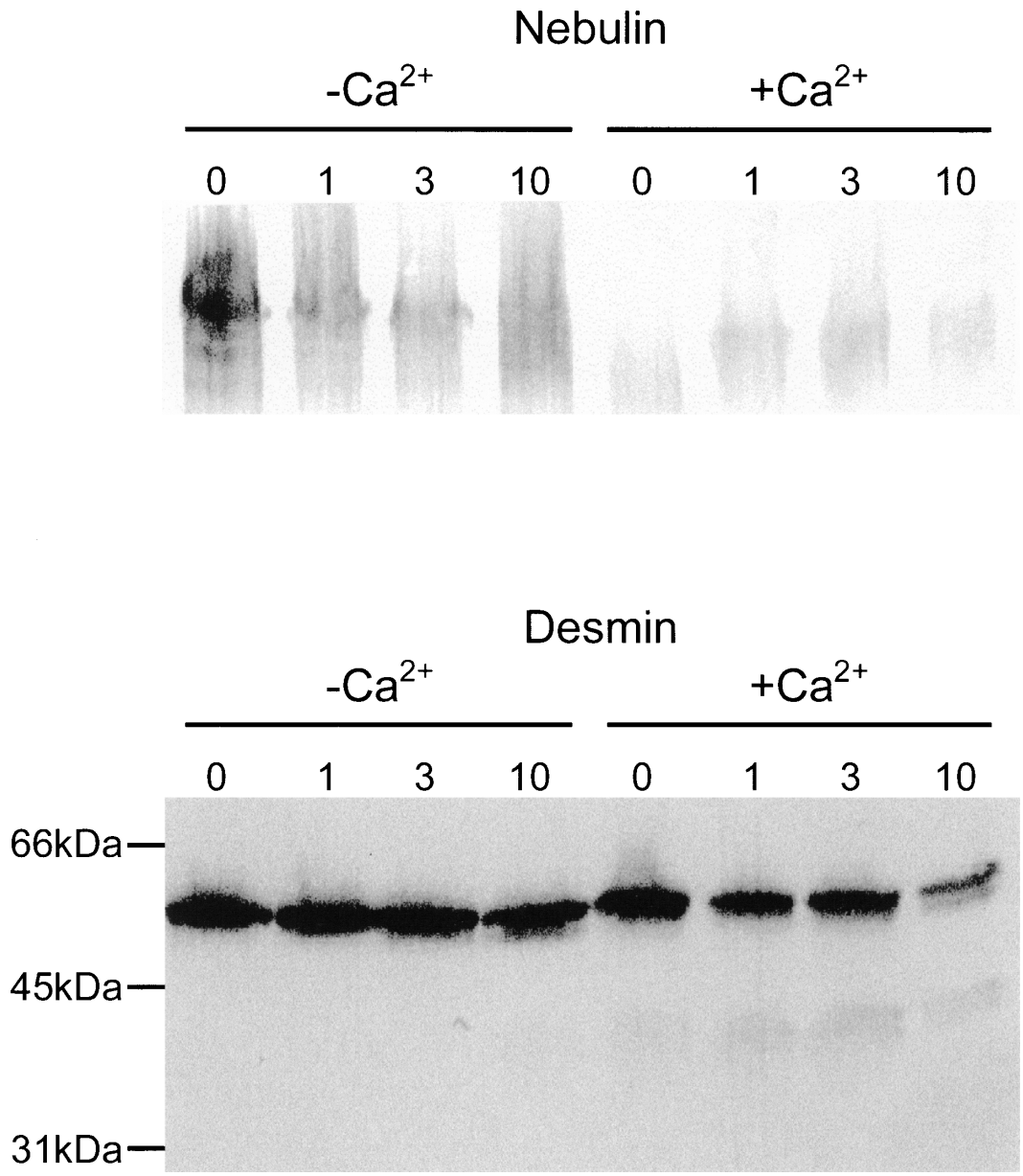

Figure 2. Western blots using antibodies to nebulin (upper panel) or desmin (lower panel) of myofibrils obtained from longissimus muscle after different times of postmortem storage. Myofibrils that had been washed three times (Materials and Methods) were incubated for 48 h at 15°C in 0.2 m*M* EGTA (minus Ca^{2+}) or 10 m*M* Ca^{2+} as indicated. After 48 h, the myofibrils were sedimented at 16,000 × g_{max} for 5 min and suspended in SDS-SB. Myofibrils were from muscle after 0, 1, 3, or 10 d of postmortem storage as indicated. All lanes were loaded with 20 μg of myofibrillar protein per lane. Arrows indicate the migration distances of polypeptides of the molecular weights specified.

effect on degradation of troponin T (Figure 4), autolysis of myofibril-bound μ-calpain (Figure 5), and release of intact α-actinin (Figure 6).

Discussion

It is now well established that the calpain system is responsible for most if not all the proteolysis of myofibrillar proteins that occurs during postmortem storage at 2 to 4°C (Koohmaraie, 1988, 1994, 1996). Although it has remained unclear how the calpains function in postmortem muscle (Taylor et al., 1995; Goll et al., 1998; Geesink and Koohmaraie, 1999a), the discovery of myofibril-bound calpain offered an attractive new possibility for the action of calpains during postmortem storage. The nature of the myofibrillar-bound proteolytic activity remains unclear despite the information obtained in this study. First, dot-blot analyses indicated that approximately 40% of total μ-calpain was bound to the myofibril after 1 d of postmortem storage, and it was estimated that 1-d postmortem myofibrils contain 0.13 μg of μ-calpain protein per milligram of myofibrillar

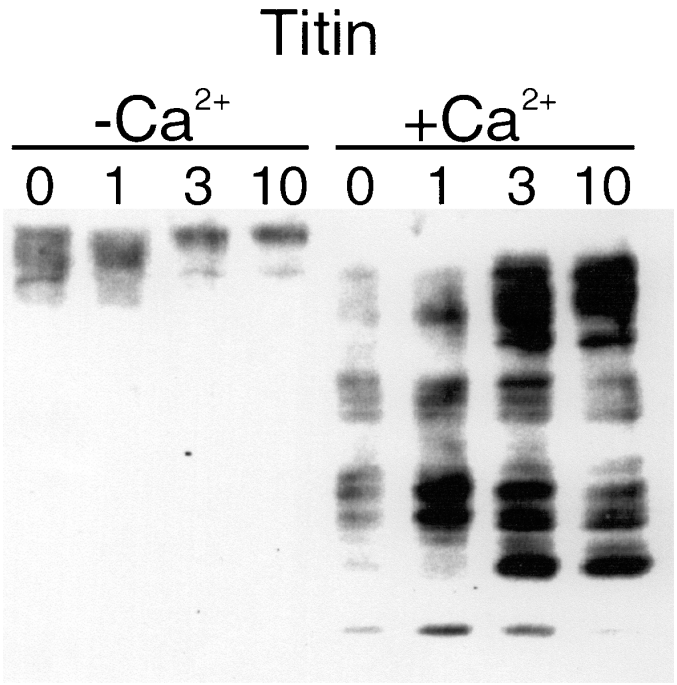

Figure 3. Western blots using antibodies to titin of myofibrils obtained from longissimus muscle after different times of postmortem storage. Myofibrils that had been washed three times (Materials and Methods) were incubated for 48 h at 15°C in 0.2 m*M* EGTA (minus Ca^{2+}) or 10 m*M* Ca^{2+} as indicated. After 48 h, the myofibrils were sedimented at 16,000 × g_{max} for 5 min and suspended in SDS-SB. Myofibrils were from muscle after 0, 1, 3, or 10 d of postmortem storage as indicated. All lanes were loaded with 18 mg of myofibrillar protein. The giant titin polypeptide at the top of the gel is completely degraded in all the myofibrils after 48 h in 5 m*M* Ca^{2+}.

protein. Based on these estimates and assays of the myofibrillar-bound proteolytic activity, it can be calculated that total myofibrillar-bound proteolytic activity was 1.6×10^{-2} cpm per gram of muscle. The extractable μ-calpain activity from longissimus was 0.377 unit per gram of muscle (Delgado et al., 2001). One unit of calpain activity is 2.6×10^6 cpm, so even assuming that all of the myofibrillar proteolytic activity originates from μ-calpain, the myofibrillar bound proteolytic activity accounts for only 4% of total μ-calpain activity, although 40% of total μ-calpain protein is bound to the myofibril. These estimates obviously are based on several approximations, but they agree closely with earlier results indicating that approximately 35% of total μ-calpain protein was bound to 1-d postmortem myofibrils from bovine semimembranosus muscle (Boehm et al., 1998). Assays of myofibrillar-bound proteolytic activity in bovine muscle, however, indicated that only 7 to 8% of total μ-calpain activity was associated with the myofibrils (Boehm et al., 1998). Together, the results indicate that specific activity of the myofibrillar-bound proteolytic activity, assuming it originates entirely from μ-calpain, is approximately only 6 to 13% as high as the specific activity of the extractable μ-calpain from the same muscle.

It is possible that this low specific activity is a result of the way activity was assayed. The μ-calpain evidently is tightly bound to the myofibril, indicating that the off rates for its dissociation from the myofibril are so low that the molecules are bound to the myofibril most of the time. If the active site of the bound molecule is not available to degrade an exogenous casein substrate, the rates of proteolysis as measured in our assays (and in those of Boehm et al., 1998) would be very low. On the other hand, with the possible exception of nebulin and titin, the myofibrillar proteins were not extensively degraded considering the long incubation times and the large amounts of μ-calpain protein present. It is possible that the myofibrillar bound μ-calpain is active in postmortem muscle but that its activity is restricted to those areas to which it is bound and that these areas are not widespread. A careful immunoelectron localization of the myofibril-bound μ-calpain will be required to answer these questions.

Second, it is unclear why the inhibitors added to the 1-d postmortem myofibrils did not completely inhibit the myofibrillar-bound proteolytic activity. The inhibitors were added in large molar excess and were able to completely inhibit μ-calpain added exogenously to the

Table 4. Effect of three cysteine protease inhibitors on myofibril-bound proteolytic activity of callipyge and normal longissimus dorsi muscle[a]

Sample	Without inhibitor	E-64[b]		Leupeptin (Leup.)[b]		Iodoacetate (IAA)[b]	
		With E-64	%In[c]	With leup.	%In	With IAA	%In
Callipyge	18.7	6.5	65%	2.9	84%	8.0	57%
Normal	16.4	5.7	67%	2.0	89%	7.0	58%
μ-Calpain[c]	50.6	1.5	97%	3.7	93%	3.4	93%

[a]Numbers are counts of ^{14}C released from ^{14}C-labeled casein per microgram of myofibrillar protein after incubation with myofibrils that had been prepared from 1-d postmortem longissimus muscle for 48 h at 15°C. Numbers are means of determination done on each of six animals (12 total assays per mean). Final molar concentration of potassium chloride in all assays was 0.1 *M*.
[b]Final concentrations in the assay were: E-64, 0.33 *M*; leupeptin, 1.50 m*M*; iodoacetate, 0.41 m*M*.
[c]%In: Percentage inhibition.
[d]Purified μ-calpain from bovine lung, final concentration in assay was 893 ng protein/mL (8.1 n*M*).

Table 5. Effect of calpastatin on myofibril-bound proteolytic activity[a]

Sample	Without calpastatin[b]	With calpastatin	Inhibition, %
Callipyge	25.3	14.6	42.9
Normal	22.5	12.3	45.3
μ-Calpain[c]	41.6	1.4	96.7

[a]Myofibrils were prepared from 1-d postmortem longissimus muscle, and assays were done as described in the Materials and Methods. Numbers are counts per minute per microgram of myofibrillar protein obtained after 5 min of counting and subtraction of the Ca^{2+}-free blank. Numbers are means of two determinations done on each of six animals (12 total assays per mean). The standard error was 3.1. There were no significant differences between the normal and callipyge sheep.

[b]Purified bovine skeletal muscle calpastatin; 2.4 units/mL.

[c]Purified μ-calpain from bovine lung; final concentration in the assay was 8.1 n*M*.

assay. That leupeptin, a broader spectrum inhibitor, was slightly more effective than E-64 or iodoacetate might suggest that some of the myofibrillar-bound proteolytic activity originated from a Ca^{2+}-activated serine protease. Autolysis of the bound μ-calpain and release of undegraded α-actinin, however, are reasonably specific properties of the calpains (the autolysis would likely have had to have been intramolecular because of the tight binding), and no known Ca^{2+}-activated serine protease would cause such autolysis or release of undegraded α-actinin. Consequently, it remains highly possible that the active site of myofibrillar-bound μ-calpain is masked and is unavailable or only partly available to active site-directed inhibitors. That calpastatin, which is a much larger molecule than the other inhibitors tested, was also the least effective is consistent with this concept. Hence, additional studies will be needed to distinguish among several remaining possibilities: 1) that the myofibrillar-bound proteolytic activity is due almost entirely to tightly bound μ-calpain whose active site is partially blocked, so that it does not degrade exogenously added substrates such as casein and inhibitors can only partly inactivate it; 2) that approximately 40 to 50% of the myofibrillar-bound proteolytic activity is due to bound μ-calpain that is inhibited by calpastatin and other calpain inhibitors and that the remaining 50 to 60% of the myofibrillar-bound proteolytic activity is due to some, as yet unidentified, Ca^{2+}-activated protease that is inhibited by leupeptin, but not by calpastatin and weakly by E-64 or iodoacetate; or 3) that the myofibril-bound proteolytic activity originates from a partly inactivated/denatured μ-calpain that has adventitiously bound to the myofibril after being autolyzed and that is only partly affected by its usual inhibitors.

Finally, the myofibril-bound proteolytic activity in 1-d longissimus muscle was the same in the callipyge and normal sheep even though the Warner-Bratzler shear force values for the callipyge longissimus muscle were nearly double the shear force values for the normal longissimus (Delgado et al., 2001). Consequently, it seems unlikely that myofibrillar-bound proteolytic activity has an important role in postmortem tenderization, at least in the sheep longissimus. Although it could be argued that the large amount of calpastatin in callipyge longissimus would negate any effect that the myo-

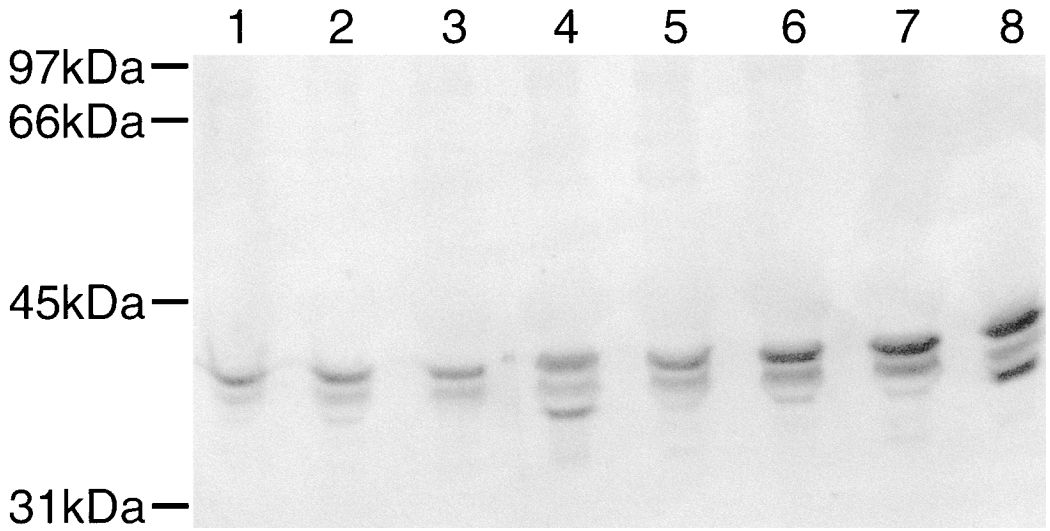

Figure 4. Western blots using anti-troponin T antibodies of myofibrils obtained from 1-d postmortem longissimus muscle and incubated for 48 h at 15°C in the absence or presence of protease inhibitors. Lane 1, not incubated; Lane 2, preincubated for 42 h without Ca^{2+} or EGTA; Lane 3, preincubated for 42 h without Ca^{2+} or EGTA and then incubated for 48 h with 0.2 m*M* EGTA added after the first 42 h; Lane 4, same as Lane 3 except 10 m*M* Ca^{2+} added after the 42 h preincubation; Lane 5, same as Lane 4 but with 0.33 m*M* E-64 present; Lane 6, same as Lane 4 but with 1.50 m*M* leupeptin present; Lane 7, same as Lane 4 but with 0.41 m*M* iodoacetate present; Lane 8, same as Lane 4 but with 0.4 unit of calpastatin present. Arrows indicate location of molecular weight markers. All lanes were loaded with 20 μg of myofibrillar protein per lane.

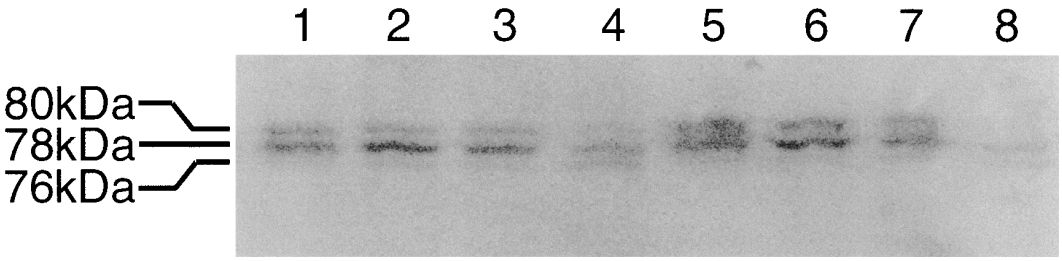

Figure 5. Western blots using anti-μ-calpain antibodies of myofibrils obtained from 1-d postmortem longissimus muscle and incubated for 48 h at 15°C in the presence or absence of protease inhibitors. Lane 1, not incubated; Lane 2, preincubated for 42 h without Ca^{2+} or EGTA; Lane 3, preincubated for 42 h without Ca^{2+} or EGTA and then incubated for 48 h with 0.2 m*M* EGTA added after the first 42 h; Lane 4, same as Lane 3 but 10 m*M* Ca^{2+} added after the first 42-h preincubation; Lane 5, same as Lane 4 but with 0.33 m*M* E-64 present; Lane 6, same as Lane 4 but with 1.50 m*M* leupeptin present; Lane 7, same as Lane 4 but with 0.41 m*M* iodoacetate present; Lane 8, same as Lane 4 but with 0.4 unit of calpastatin present. Arrows indicate location of molecular weight markers. All lanes were loaded with 150 μg of myofibrillar protein per lane.

fibril-bound proteolytic activity might have in postmortem proteolysis, the discussion in the previous paragraph indicates that the myofibrillar proteolytic activity is not inhibited or only partly inhibited by a large excess of calpastatin. Hence, it is unlikely that the increased calpastatin in the callipyge longissimus would have any appreciable effect on myofibrillar-bound proteolytic activity, or that the myofibrillar-bound proteolytic activity has an important role in postmortem tenderization in callipyge sheep longissimus.

Implications

Discovery that a significant proportion of μ-calpain becomes associated with the myofibril during postmortem storage raised an attractive new explanation for the mechanism of postmortem tenderization. The myofibril-bound μ-calpain would be located directly on the substrates that it degrades and whose degradation leads to increased tenderness. Moreover, the myofibril-bound calpain had been reported to be resistant to inhi-

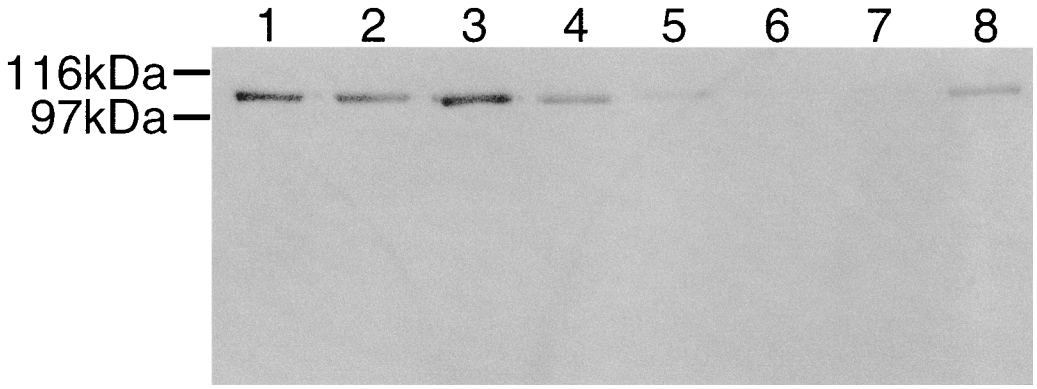

Figure 6. Western blots using anti-α-actinin antibodies of the supernate after sedimentation of myofibrils from 1-d postmortem longissimus muscle. Myofibrils were preincubated for 42 h at 5°C in 100 m*M* KCl, 20 m*M* K-phosphate, pH 5.8, 2 m*M* $MgCl_2$, 2 m*M* EGTA, 1 m*M* NaN_3 (standard salt solution) and were then incubated for 48 h at 15°C in the presence or absence of different protease inhibitors. After incubation, the myofibrils were sedimented at 16,000 × g_{max} for 5 min, the supernate was removed and mixed with Laemmli buffer (2 vol supernate:1 vol buffer), and 18 μg of protein were loaded onto each lane in the gel. Lane 1, not incubated; Lane 2, preincubated for 42 h in standard salt solution; Lane 3, preincubated for 42 h in standard salt solution, and then incubated for 48 h with 0.2 m*M* EGTA added to the standard salt solution; Lane 4, same as Lane 3 but with 10 m*M* $CaCl_2$ added after the first 42-h preincubation; Lane 5, same as Lane 4 but with 0.33 m*M* E-64 present; Lane 6, same as Lane 4 but with 1.5 m*M* leupeptin present; Lane 7, same as Lane 4 but with 0.41 m*M* iodoacetate present; Lane 8, same as Lane 4 but with 0.4 unit of calpastatin present. Arrows indicate the position of molecular weight markers. All lanes were loaded with 18 μg of protein.

bition by calpastatin, so it could be active in postmortem muscle in the presence of excess calpastatin. Results from this study show that the myofibril-bound calpain activity in the longissimus muscle from callipyge and normal sheep does not differ. Because the shear force values for callipyge longissimus are twice as high as for longissimus from normal sheep, it seems unlikely that the myofibrillar-bound calpain activity has a significant role in postmortem tenderization of sheep longissimus.

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